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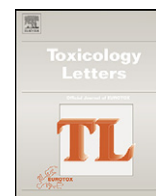
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## Abnormal expression of MAPK, EGFR, CK17 and TGk in the skin lesions of chloracne patients exposed to dioxins

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### ABSTRACT

**Objective:** Chloracne is one of the most sensitive and specific hallmark of dioxin intoxication. Although its clinical features are clearly described, poor understanding of the molecular pathways of dioxin-induced chloracne hampers a rational approach to therapy. The aim of the present study was to investigate the role of EGFR, MAPK, CK17, and TGk in the pathogenesis of chloracne related to dioxin exposures.

**Methods:** Epidermal tissues of twelve chloracne patients exposed to dioxins were compared with tissues from 12 healthy controls. These skin tissues were obtained by punch biopsies. p-EGFR and p-MAPK were examined by immunofluorescence. The mRNA and protein levels of CK17 and TGk were examined by fluorescence *in situ* hybridization and immunohistochemistry, respectively.

**Results:** p-EGFR and p-MAPK were found in all chloracne tissues, whereas no expression was found in the controls. CK17 mRNA and protein were also found in all chloracne lesions, but none in controls ( $P=0.000$ ). TGk mRNA and protein were detected in both groups, but the distribution was distinct. The positive signals in the controls were mainly in the stratum granulosum, while in the chloracne tissues, the positive signals were found more significantly in the stratum granulosum and stratum spinosum.

**Conclusions:** The results demonstrate that in the human skin the activation of mitogen-activated protein kinase pathway and up-regulation of CK17 and TGK may play roles in the pathogenesis of chloracne related to dioxin exposures.

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### 1. Introduction

In recent years, a great number of studies have been focused on the global environmental pollution caused by dioxins, such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs). Dioxins are lipophilic, persistent, bioaccumulative, and toxic (Kawashima et al., 2006). The most biologically active isomer is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which can elicit

a wide spectrum of toxic effects in humans, including reproductive and developmental toxicity, immunotoxicity, epithelial disorders as well as carcinogenicity (Huang et al., 2002; Sweeney and Mocarelli, 2000).

Chloracne, a specific type of acne-like dermatosis, is caused by exposure to certain halogenated polycyclic hydrocarbons such as TCDD, and considered to be one of the most sensitive and specific biomarkers of TCDD intoxication (Coenraads et al., 1999; Suskind, 1985). Chloracne is a well-recognized clinical entity characterized by an acne-like eruption of blackheads and whiteheads, epidermal cysts, pustules and papules (Panteleyev and Bickers, 2006). Chloracne lesions are especially found on the face, neck, earlobes, shoulders, abdomen, legs, and genitalia. The most commonly affected areas are in the malar crescent and postauricular triangles. Although the clinical features of chloracne are clearly described, a rational approach to therapy is hampered by a poor understanding of its pathogenesis and molecular pathways.

**Abbreviations:** EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; CK17, cytokeratin 17; TGk, transglutaminase 1; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aromatic hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; AP-1, activator protein-1; PTK, protein tyrosine kinase.

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The pathogenesis of chloracne caused by TCDD probably involves many reactions that are related to the cell proliferation and differentiation. Some investigators have reported that TCDD up-regulates the mRNA levels of *c-fos* and *c-jun* in mouse hepatoma cells, and concomitantly increase DNA-binding activity of the transcription factor AP-1, a dimer of *c-fos* and *c-jun* proteins, which does not require a functional AhR (Puga et al., 1992). The direct activation without ARNT (aryl hydrocarbon nuclear translocator) function of the protein kinase cascade by TCDD/AhR complex (Enan and Matsumura, 1996) may represent a molecular mechanism for the induction and development of chloracne by TCDD because its downstream targets such as EGFR are known to be involved in regulating epithelial cell proliferation and differentiation (Hirano et al., 2000; Jensen et al., 2000; Mimeault et al., 2004; Murphy et al., 2004; Panteleyev et al., 1997; Ray and Swanson, 2003). These results imply that the mitogen-activated protein kinase (MAPK) pathway might be involved in the TCDD-induced chloracne.

The MAPK pathway may alter the expression of a number of genes related to proliferation and differentiation, and a wide range of growth factors, such as epidermal growth factor (EGF). EGF receptor signaling blocks aryl hydrocarbon receptor-mediated transcription and cell differentiation in human epidermal keratinocytes (Sutter et al., 2009). Keratin gene expression is also highly regulated. The expression of keratins has been studied in normal and pathological keratinocyte proliferation and differentiation (Chu and Weiss, 2002). Keratin 17, which is not expressed in normal skin, is over-expressed in chloracne lesions (Panteleyev et al., 1997). Keratinocyte transglutaminase (TGK) is a member of the transglutaminase family the homeostasis of which is maintained by proliferation and terminal differentiation of keratinocytes and squamous metaplasia (Roop, 1995; Ta et al., 1990).

To shed light on the molecular mechanisms of dioxin-induced chloracne, we investigated the expression and distribution of phosphorylated epidermal growth factor receptor (p-EGFR), phosphorylated mitogen-activated protein kinase (p-MAPK), cytokeratin 17 (CK17), and transglutaminase 1 (TGK) in the skin tissue of chloracne patients and healthy control subjects.

## 2. Subjects and methods

### 2.1. Study subjects

We recruited 12 male chloracne patients who had worked in a Chinese factory where pentachlorophenol (PCP) was produced from hexachlorocyclohexane (HCH) before 2002. It has been shown that dioxins are impurities that are formed during the production process of PCP (Cheng et al., 1993). The 12 chloracne cases were similar in severity of their visible clinical manifestations. Although they had been away from the PCP production since 2002, the patients still suffered from chloracne during the sample collection. A number of these chloracne cases and their exposure to dioxins have been described previously. The blood lipid concentration of TCDD TEQ necessary to develop chloracne is apparently between 650 and 1200 pg/g, approximately more than 10 times the WHO reference background level (Coenraads et al., 1999). Controls were 12 healthy age-matched males with no history of such kind of exposure, had no other skin disease and were from the same factory. In our study, drinkers were those who used alcohol (that is, wine, beer, or hard liquor) on 3 or more days per week. Those who indicated that they were occasional or party drinkers were not counted as drinkers in this study. A smoker was defined as a person who smoked at least 5 cigarettes daily for more than one year (Doll and Hill, 1999).

All study subjects answered an occupational questionnaire and gave informed consent. The research protocol was approved by the Ethics and Research Committees of all participating institutions, and followed the tenets of the Declaration of Helsinki.

### 2.2. Sample collection

According to the routine dermatological practice procedures, local anesthesia with lidocaine was used in all subjects. The epidermal tissues were obtained by punch biopsies (Biopsy Punch, Waechtersbach, Germany) from the neck, back, and face for 3, 5, and 4 chloracne patients, respectively. The samples from the face were obtained by a fusiform incision in order to be sutured easily and to guarantee acceptable cosmetic appearance. All these 12 biopsies were from lesional skin. The tissues for the controls were taken from the same localizations by the same

process. Every piece of skin was 5 mm × 2 mm in diameter × thickness. All biopsies were performed by an experienced surgeon. Immediately after the collection, the fresh biopsy samples were briefly washed in saline, snap-frozen and stored in liquid nitrogen.

### 2.3. Immunofluorescence for p-MAPK and p-EGFR

p-MAPK and p-EGFR were detected by indirect immunofluorescence. Skin samples were cut in a cryostat at −20 °C, dried and blocked with goat serum for 20 min at room temperature, and then incubated for 2 h at 37 °C with the primary antibody for either p-MAPK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or p-EGFR (Cell Signaling Technology, USA). After washing with phosphate-buffered saline (PBS), the slides were incubated for 1 h at 37 °C with fluorescein isothiocyanate-labelled secondary antibodies (Santa Cruz Biotechnology, Inc./Cell Signaling Technology, USA), washed again with PBS four times for 5 min each. The samples were mounted and then examined by confocal laser scanning microscopy (Bio-Rad, Deisenhofen, Germany).

### 2.4. Fluorescence in situ hybridization for CK17 and TGK expression

All skin biopsies of the chloracne group and the control group were serially sectioned at 8 μm. The slides were treated with polyoxymethylene and citric acid buffer solution for 10 min, and then incubated with the same buffer containing protease K and pepsin at 37 °C for 10 min. After washing with PBS three times for 5 min and once with 0.2 × SSC (sodium chloride–sodium citrate buffer) for 3 min, the slides were incubated in a prehybridization solution (*in situ* hybridization solution without probe and dextran sulphate) for 4 h in a wet box at 42 °C, and then washed again with 2 × SSC three times for 5 min and with 0.2 × SSC three times for 5 min. After that, the slides were incubated with *in situ* hybridization solution containing direct fluorescent probe and covered with cover slips for hybridization. The hybridization was kept in a wet box at 42 °C overnight; the sections were then taken out and washed three times with 2 × SSC for 5 min, 0.2 × SSC three times for 5 min and PBS three times for 5 min. The washed sections were mounted with glycerol and examined by confocal laser scanning microscopy (Bio-Rad, Deisenhofen, Germany).

The sequences of probes were as follows (Hao Yang Biological Manufacture Co., Ltd., China):

CK17 (5′–TGCCA TAGCC ACCAC CAGAG CCAAA GC)  
TGK (5′–TACTC ATACT CGTCT GTGTG GTGCT CTCGG)

### 2.5. Immunohistochemistry for CK17 and TGK

Skin sections were rinsed in PBS three times for 5 min. After treatment with hydrogen peroxide for 10 min and goat serum for 1 h, the samples were incubated for 2 h at 4 °C with antibodies. Working concentration of both anti-CK17 (Zhongshan Goldenbridge Biotechnology Co., Ltd., China) and anti-TGK (Biomedical Technologies Inc., USA) were 1:20. After the incubation, the slides were washed with PBS three times for 5 min. The slides were mounted and then examined by microscopy.

### 2.6. Statistical analysis

Statistical analysis of the comparison of the presence/absence of the expression of p-EGFR, p-MAPK, CK17 between the cases and the controls was by Chi-square test, with a *P*-value of 0.05 level of significance.

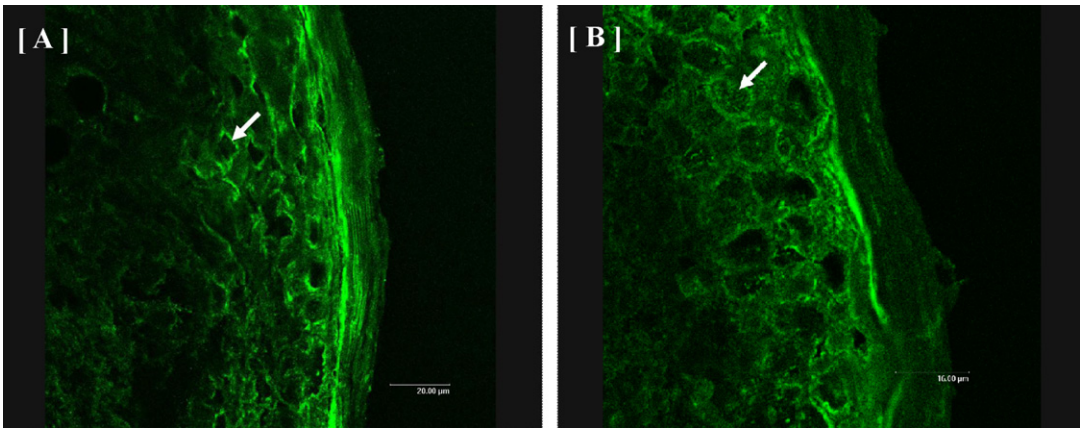
## 3. Results

### 3.1. Basic demographic characteristics of cases and controls

Cases (*n* = 12) and controls (*n* = 12) were all males, and were comparable in age (52 ± 7 years vs. 52 ± 5 years), body mass index (BMI) (23.54 ± 2.48 vs. 24.72 ± 2.37), and smoking and drinking habits (Table 1).

### 3.2. Expression of p-EGFR and p-MAPK in epidermis

p-EGFR and p-MAPK were found in all chloracne lesions, whereas no expression was found in the samples from the control group (*P* = 0.000) (Fig. 1). In the skin of the chloracne patients, p-EGFR was mainly distributed in the membrane and cytoplasm, especially in the vicinity of membrane; the major positive signal of p-MAPK was in the core and serosity.



**Fig. 1.** Immunofluorescence of EGFR, MAPK in the lesional skin of a chloracne patient. Phosphorylated EGFR [A] was mainly distributed in the membrane and the cytoplasm, especially in the vicinity of membrane; phosphorylated MAPK [B] was mainly in core and serosity, which are indicated by the arrow.

**Table 1**  
Demographic characteristics of controls and chloracne cases.

	Controls (n = 12)	Chloracne cases (n = 12)	P-value
Age (years) <sup>a</sup>	51.42 ± 5.30	52.08 ± 7.62	0.806
BMI (kg/m <sup>2</sup> ) <sup>a</sup>	24.72 ± 2.37	23.54 ± 2.48	0.246
Smoker <sup>b</sup>			
Yes	9 (75.0)	11 (91.7)	0.590
No	3 (25.0)	1 (8.3)	
Alcohol intaker <sup>b</sup>			
Yes	10 (83.3)	8 (66.7)	0.640
No	2 (16.7)	4 (33.3)	

<sup>a</sup> Mean ± SD, analyzed by Student's *t*-test.  
<sup>b</sup> *n* (%), analyzed by Fisher's exact test.

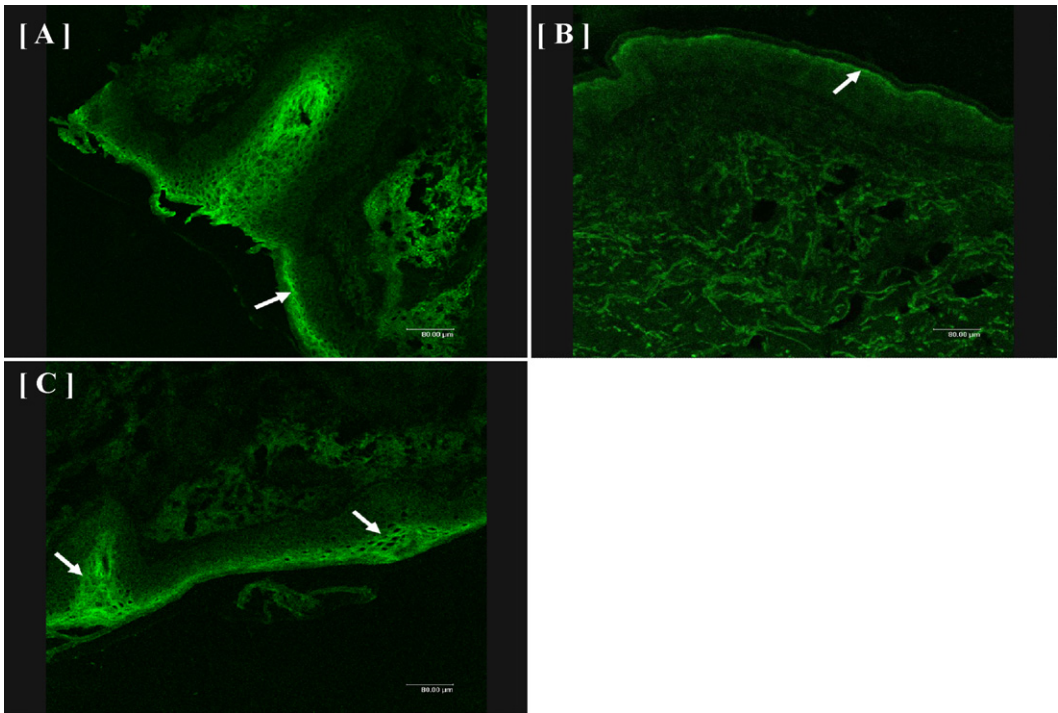
3.3. Gene and protein expression of CK17 and TGk

CK17 mRNA and protein were also found in the stratum spinosum in all chloracne lesions (Figs. 2 and 3), whereas no expres-

sion of it was found in any skin sample from the control group (*P*=0.000) (Figs. 2 and 3). The expression of TGk mRNA and TGk protein could be found both in the skin of chloracne patients and in tissues of normal controls. In normal controls, the positive signals were found mainly in the stratum granulosum (Figs. 2 and 3). In the chloracne lesions, the positive signals were found more significantly in the stratum granulosum and stratum spinosum (Figs. 2 and 3).

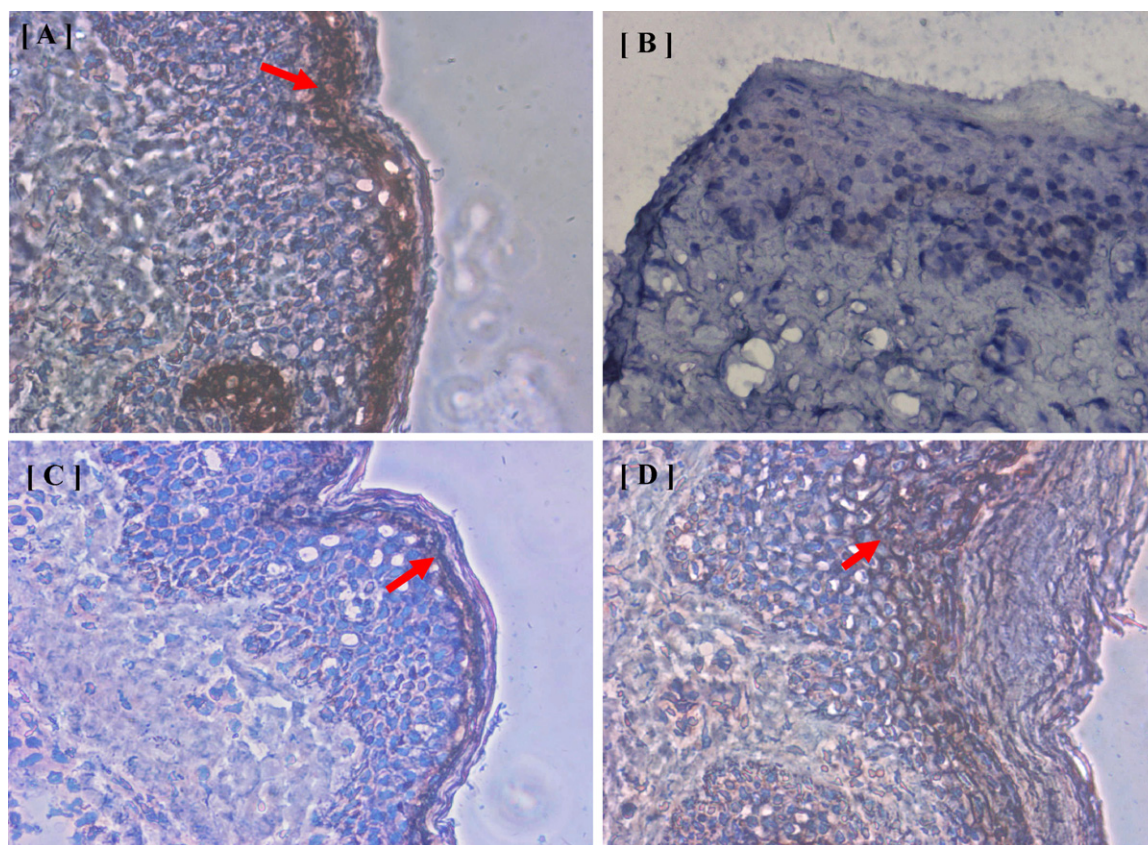
4. Discussion

TCDD, the most biologically active isomer of dioxins, is generally considered to be the most potent man-made toxicants. The molecular mechanism of the effects of TCDD is complicated and remains unclear, while one biological effect of TCDD is mediated through the aromatic hydrocarbon receptor (AhR) (Dhooge et al., 2006). Chloracne, the typical skin disorder, is considered to be



**Fig. 2.** [A] CK17 mRNA in the stratum spinosum of a chloracne lesion (no expression in any skin sample from the control group). [B] Expression of TGk mRNA in the skin of a control sample, mainly in the stratum granulosum. [C] Expression of TGk mRNA in the lesional skin of chloracne patients, mainly in stratum granulosum and stratum spinosum. The arrows indicated the expression of Ck17 and TGk mRNA.





**Fig. 3.** [A] Chloracne lesion: CK17 in the stratum spinosum. [B] Control: no expression of CK17 in any sample. [C] Control: expression of TGk mainly in stratum granulosum. [D] Chloracne lesion: TGk more significant in stratum granulosum and stratum spinosum. Differences of the distribution of TGk are indicated by the arrow.

a sensitive indicator of the exposure to TCDD (Loertscher et al., 2002).

In the present paper, we performed immunofluorescence techniques to detect the expression of p-EGFR and p-MAPK proteins in the epithelium of chloracne patients and healthy controls. We detected p-EGFR and p-MAPK in all chloracne lesions, whereas no expression of p-EGFR and p-MAPK protein was found in the control group. Our results demonstrated that the MAPK signal transduction pathway may be one important molecular mechanism of chloracne. It has been suggested that dioxin binding to AhR activates protein tyrosine kinase (PTK) activity, and then gradually enhances the RAS protein and MAPK phosphorylation enzyme cascade. Through MAPK phosphorylation, transcription factors such as the intranuclear activator protein-1 (AP-1) are activated (Grinkevich et al., 2008). This pathway may alter the expression of a number of genes specific to cell proliferation and differentiation, and a wide range of growth factors, such as epidermal growth factor (EGF) (Davis et al., 2003), resulting in acne-like lesions.

In the past, various experimental studies on the effects of TCDD on keratinocytes and skin have been reported (Geusau et al., 2005). Treatment of non-transformed human keratinocytes with TCDD resulted in enhanced differentiation, determined by a complex interaction such as modulation of expression of a number of growth-regulatory proteins (Gaido and Maness, 1994).

The expression of specific keratins is one of the main characteristics of mammalian epidermal keratinocyte proliferation and differentiation (Chu and Weiss, 2002; McLean, 2003). Keratin gene expression is highly regulated. Patterns of most keratins expression are well defined for normal and pathological keratinocyte proliferation and differentiation so far (Chu and Weiss, 2002). Therefore, keratin expression is to be a reliable marker to evaluate the effect of TCDD on keratinocyte physiology *in vivo*.

Keratin 17, which is not expressed in normal skin except for nail bed, hair follicle, sebaceous glands and other epidermal appendages, is over-expressed in chloracne lesions. Keratin 17 was newly expressed in the upper spinous cell layers of the interfollicular epidermis after TCDD application to hairless mouse skin (Panteleyev et al., 1997). In our study, CK17 mRNA and CK17 protein was found in all chloracne lesions, whereas no expression of them was found in the control group. This expression could be found in the stratum spinosum in all chloracne lesions, demonstrating that TCDD can affect keratinocyte proliferation and differentiation.

Transglutaminases (TG) are a superfamily of structurally and functionally related enzymes that catalyze transamidation of glutamine residues, a reaction associated with a wide variety of physiologic processes such as cell differentiation, tissue regeneration, and plant pathogenicity (Aeschlimann and Paulsson, 1994; Greenberg et al., 1991). Keratinocyte transglutaminase (TGk), a member of the transglutaminase family, is expressed in upper layers of normal epidermis, the homeostasis of which is maintained by proliferation and terminal differentiation of keratinocytes and squamous metaplasia (Roop, 1995; Thacher and Rice, 1985).

In this study, we found TGk mRNA and TGk protein mainly in the stratum granulosum in normal controls. However, in the chloracne lesions, the positive signals were found more significantly in the stratum granulosum and stratum spinosum. From our experiments, we conclude that TCDD stimulates epidermal differentiation by up-regulating TGK expression.

Our study was relatively small (12 cases and 12 controls) and a skin biopsy from non-lesional adjacent skin was not obtained because it was, unfortunately, impossible to obtain consent from the affected workers for a second biopsy from their normal skin. In the future, one study should involve examining these factors in the skin lesions of a larger number of individuals with chloracne

of varying severity and in normal adjacent skin. Polymorphisms in EGFR influencing susceptibility to chloracne could also be investigated.

In conclusion, we observed an association of chloracne with the upregulation of certain genes and protein expression. We propose that MAPK signal transduction pathway plays a critical role in the formation of chloracne lesions. Since chloracne is one of the most sensitive and specific indicator of dioxin exposure, the alterations of gene and protein expression that we described in chloracne patients could provide important clues for the study of epithelial changes induced by dioxins.

### Conflict of interest statement

No competing interests.

### Acknowledgements

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